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Calcium Speciation and Exchange Between Blood and Extrapallial Fluid of the Quahog *Mercenaria mercenaria* (L.)

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Abstract. Calcium and small organic molecules (*e.g.*, tyrosine, MW 181 Da) introduced into the extrapallial fluid (EPF) of the quahog *Mercenaria mercenaria* exhibit rapid fluxes across the outer mantle epithelium and are distributed throughout the circulatory system within 3 h. Larger molecules (*e.g.*, bovine serum albumin, MW 66,000 Da) are less readily exchanged between EPF and blood. The protein compositions of blood plasma and EPF are different, with at least seven protein bands expressed more prominently in the EPF. Equilibrium dialysis experiments reveal that Ca^{2+} constitutes only 2% of the total Ca in plasma; most of the Ca (85%) is bound to macromolecules, and the remaining 13% is present as dialyzable low molecular weight moieties. This distribution cannot be explained by speciation of inorganic Ca alone, since the MINTEQA2 equilibrium speciation model predicts that 79%–86% of the Ca should be present as Ca^{2+}, with the remainder as $CaSO_4$ (20%–13%). However, inclusion of a weakly Ca-binding organic molecule ($\log_{10} K_a \approx 2$ M^{-1}) into MINTEQA2 could fully reconcile modeling with experimental measurements. Results suggest that calcium transport in blood plasma and EPF is mediated by a suite of proteins and small organic ligands with a low affinity for Ca.

Introduction

A variety of invertebrates secrete $CaCO_3$ exoskeletons. Unraveling the mechanisms involved in these secretory processes requires an understanding of the microenvironment within the compartment immediately adjacent to the site of $CaCO_3$ incorporation. The molluscan shell-formation system, for example, consists of a series of compartments, the most important of which are the inner shell surface, the extrapallial space, and the outer mantle epithelium (Crenshaw, 1980). Extrapallial fluid (EPF), a blood-like fluid contained in the extrapallial space, is considered to be the microenvironment for the deposition of shell (Wilbur, 1972; Marsh and Sass, 1983; Saha *et al.*, 1988) by an organic matrix-mediated process (Weiner, 1984; Wheeler *et al.*, 1988; Keith *et al.*, 1993). In bivalve molluscs, the muscular attachment of the mantle to the shell along the pallial line further divides the shell-forming compartment into two distinct zones. The marginal zone, outside the pallial line, is associated with the highest rate of shell deposition and contributes to increases in the height and length of the shell. The central zone, within the pallial line, is associated with both deposition (thickening) and redissolution of shell (Wheeler *et al.*, 1975; Hudson, 1992).

The EPFs associated with the marginal and central zones may have different chemical composition, in keeping with the functional differences between the two zones (Wilbur and Saleuddin, 1983). Studies on the composition of EPF have concentrated almost exclusively on the fluid from the central zone because the quantities of EPF available from the marginal zone are minuscule, making it difficult to obtain samples (Crenshaw, 1980). The central zone EPF contains a complex mixture of inorganic ions (Crenshaw, 1972a; Wada and Fujinuki, 1976) and organic components including amino acids, proteins, acid mucopolysaccharides, carbohydrates, and probably lipids (Misogianes and Chasteen, 1979; Marsh and Sass, 1983; Wilbur and Bernhardt, 1984). One or more of these com-

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ponents constitute the organic matrix, which in turn is considered to regulate $CaCO_3$ crystal nucleation, morphology, orientation, density, and growth (Wheeler and Sikes, 1984; Wheeler *et al.*, 1988; Kawaguchi and Watabe, 1993; Falini *et al.*, 1996).

Extrapallial fluid and blood are thought to be separate compartments, a conclusion based on differences in their inorganic composition (Crenshaw, 1972a). Nevertheless, inorganic ions in the EPF are probably derived from blood, while the origin of its organic constituents is still a matter of debate. The outer mantle epithelium is known to synthesize and secrete proteins, enzymes, and some organic matrix precursors into the EPF (Wilbur, 1972; Wheeler and Harrison, 1982; Saleuddin and Kunigelis, 1984; Bielefeld *et al.*, 1993; Miyamoto *et al.*, 1996). It is possible that some constituents are also synthesized elsewhere and transported into the EPF by the blood (Wilbur, 1972; Wilbur and Saleuddin, 1983). Little work has been done on the exchange of organic compounds between the blood and EPF of bivalves.

Ions involved in shell formation (Ca^{2+}, HCO_3^-, and CO_3^{2-}) are apparently freely exchanged between the EPF, the blood, and the external medium (Greenaway, 1971; Wilbur and Saleuddin, 1983). Fluxes of these ions between the EPF and blood are probably bidirectional, and occur through the outer mantle epithelium. Studies on calcium movement across the epithelia of various species indicate the existence of two major pathways, namely paracellular (Neff, 1972; Karbach, 1992; Bielefeld *et al.*, 1993; Newman and Robinson, unpubl. data) and transcellular routes. The latter uptake route involves Ca entry by simple diffusion (Sorenson *et al.*, 1980; Coimbra *et al.*, 1988), or through channels (Beirão *et al.*, 1989; Lucu, 1994), and subsequent intracellular transport by Ca-binding proteins (Bawden, 1989; Feher *et al.*, 1992), Ca vesicles (Jones and Davis, 1982; Akins and Tuan, 1993), or as calcareous concretions (Graf and Meyran, 1983; Ziegler, 1996). Transfer of Ca between the external medium and EPF has been examined *in vivo* (Crenshaw and Neff, 1969; Akberali, 1980; Dillaman and Ford, 1982), although *in vivo* demonstrations of exchanges between EPF and blood are lacking.

The present study was designed to investigate *in vivo* the exchange of Ca^{2+} and organic compounds between the central zone EPF and the blood of the bivalve *Mercenaria mercenaria* (L.). The speciation of Ca and the protein composition of blood plasma and EPF were examined in relation to this Ca exchange.

Materials and Methods

Mercenaria mercenaria (quahogs; length 65–75 mm) were purchased locally and maintained in glass aquaria filled with aerated Instant Ocean (30 PSU), at 10°C. They were fed with mixed cultures of *Isochrysis galbana* and *Dunaliella tertiolecta* thrice weekly.

Circulation experiments

Exchange of radiolabeled compounds of different sizes between EPF and blood of the quahog were studied by a series of injection experiments. The compounds studied were the inorganic cation $^{45}Ca^{2+}$ (MW = 40 Da) as $CaCl_2$, the free amino acid ^{3}H-tyrosine (MW = 181 Da), and the macromolecule ^{14}C-bovine serum albumin (BSA; MW = 66,000 Da). Each compound was introduced into the central zone extrapallial space of the right shell. Its redistribution was monitored by periodic sampling of the EPF from the left and right sides, and blood drawn from the anterior and posterior adductor muscles.

Access to the EPF was obtained by drilling through the central zone of the shell using a Dremel high-speed cutter with a circular burr. Powdered shell generated during the drilling was removed with a suction tube connected to a vacuum pump. Drilling was stopped just before the edge of the burr penetrated the inner shell surface, and the extrapallial space was exposed by gently teasing away the innermost nacreous layer with a needle.

Injections and EPF sample withdrawals were performed using a micro-syringe with a bent needle, taking care not to damage the underlying mantle tissue. The holes in the shell were sealed with hot glue immediately after injection or sampling. Blood samples from the anterior and posterior adductor muscles were withdrawn by inserting a micro-syringe needle between the shells. Just enough shell adjacent to the adductor muscles was removed to facilitate needle insertion. Typically, each experiment consisted of injecting 10 μl of radiolabel (Ca^{2+} = 1.9, tyrosine = 0.67, and BSA = 0.025 mCi/ml) and withdrawing 50 μl of sample at intervals over a period of 4–4.5 h. The injected volume was $\leq$0.05% of the total blood volume, as determined from the relationship of shell length to blood volume for *M. mercenaria* (Robinson and Ryan, 1988).

Quahogs were held out of water during the duration of the experiment, then dissected and inspected for damage to mantle tissue. Samples from animals with damaged mantles were rejected. Blood and EPF samples thus collected were placed in borosilicate scintillation vials, mixed with 10 ml of scintillation fluid (Packard Hionic Fluor), and left overnight in the dark to reduce chemiluminescence. Radioactivity was counted using a Packard 1500 Tri-Carb liquid scintillation counter, corrected for background counts. In an additional experiment, cold BSA instead of ^{14}C-BSA was injected into quahogs (10 μl, 100 mg/ml), and EPF and blood samples were withdrawn at 3 h and 6 h. Those samples were analyzed by electrophoresis, as described below.

Calcium measurements

Shells of *M. mercenaria* (n = 10) were cracked and pried open to drain mantle cavity seawater (Robinson and Ryan, 1988). Whole blood was then collected by dissecting tissues from the shells and draining the tissues into an acid-cleaned Falcon tube over a 30-min period. Blood thus collected could be contaminated by very small amounts of EPF that had leaked through the damaged mantle. Cell-free plasma was obtained by centrifugation at 1750 × g for 10 min. 'Total Ca' in the plasma was determined by the *o*-cresolphthalein complexone method (Kessler and Wolfman, 1964; Sigma Diagnostics procedure no. 587), with absorbance measured at 575 nm using a Kontron Uvikon spectrophotometer. Optical densities were converted to m*M* using a $CaCO_3$ standard curve, which was linear between 1 m*M* and 7 m*M* (detection limit = 0.3 m*M*).

'Free Ca' (Ca^{2+}) was measured using an Orion EA 910 specific ion meter fitted with an Orion 93-20 Ca ion-specific electrode (ISE) and 90-01 single junction reference electrode. A 5-ml sample was combined with 0.1 ml of ionic strength adjustor (4 *M* KCl), continuously stirred, and maintained at 4°C. Millivolt readings were converted to m*M* Ca^{2+} using a standard curve (mv *vs.* $\log_{10}$ m*M* Ca^{2+}) prepared from $CaCO_3$, which followed the Nernst equation and had a detection limit of 0.07 m*M*. 'Bound Ca' was defined as inorganic Ca compounds and Ca bound to organic ligands (calculated as the difference between total Ca and Ca^{2+}).

Dialysis experiments

Individual blood plasma samples (3 ml; n = 10) were dialyzed against 30 ml of oyster maintenance solution (Tripp *et al.*, 1966; prepared without Ca) in Spectra/Por-7 dialysis bags (MWCO 1000 Da). Dialysis was conducted at 10°C for 48 h on a rotary shaker (100 rpm). 'Dialyzable Ca' was defined as free Ca plus Ca bound to ligands <1000 Da, which would include low molecular weight organic compounds and inorganic Ca species. 'Nondialyzable Ca' was defined as Ca bound to ligands (primarily organic) >1000 Da. At the end of dialysis, concentrations of total Ca and free Ca were measured on samples withdrawn from inside the dialysis bags (dialyzable + nondialyzable Ca) and from the external solution (dialyzable Ca). Nondialyzable Ca concentration was calculated by subtraction.

Calcium speciation modeling

Results from dialysis experiments were compared to theoretical estimates of Ca speciation by using the equilibrium speciation model MINTEQA2/PRODEFA2 (Allison *et al.*, 1991). Values for the molar concentration of the major inorganic ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, and total CO_2) in blood and EPF of *M. mercenaria* were obtained from Crenshaw (1972a). Since blood SO_4^{2-} and CO_2 concentrations were not available, the corresponding EPF values (Crenshaw, 1972a) were substituted as an approximation. The total CO_2 molarity provided by Crenshaw (1972a) was entered in the model as CO_3^{2-}. MINTEQA2 was run using various permutations of the given concentrations to understand more clearly changes in Ca speciation with changing blood and EPF composition. This included adding a hypothetical Ca-binding protein to examine organic Ca speciation. The MINTEQA2 model provided percentage distributions of free, inorganic, and organic Ca species formed under the given conditions.

Electrophoresis

Plasma and EPF were subjected to discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (1970), with a 3% stacking and 7.5% resolving gel. Protein concentrations in the samples [plasma = 1.34 ± 0.15 mg/ml, n = 5; EPF = 0.97 ± 0.17 mg/ml, n = 5; determined by Bradford's (1976) dye binding method] were adjusted by dilution such that the amount of protein applied per lane was 40–60 μg. Stacking (100 V d.c.; 2 h) and resolving (200 V d.c.; 4 h) were carried out at 15°C. Gels were stained either with 0.1% Coomassie brilliant blue R-250 (Hames and Rickwood, 1981) or with a periodic acid-silver stain for glycoproteins, as described by Dubray and Bezard (1982). Protein molecular weights were determined by comparison with a standard mixture containing myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

Results

Quahogs injected with $^{45}Ca^{2+}$ and ^{3}H-tyrosine showed a progressive decrease in radioactive counts in the right shell EPF, the site of injection (Fig. 1a, b). Concentrations of both materials decreased 3 orders of magnitude over a period of 4 to 4.5 h. Samples of blood from the anterior and posterior adductor muscles and of EPF from the left shell exhibited a corresponding increase in $^{45}Ca^{2+}$ and ^{3}H-tyrosine counts. Counts from the four sampling points converged at approximately 3 h, indicating complete mixing of the introduced radiolabel with the blood. The high molecular weight BSA, on the other hand, showed less tendency to redistribute into the circulatory system (Fig. 1c). Although a decline in BSA concentration was observed in the right EPF, a difference of 3 orders of magnitude remained between counts at the point of injection and at the three other sampling sites at 4 h post-injection.

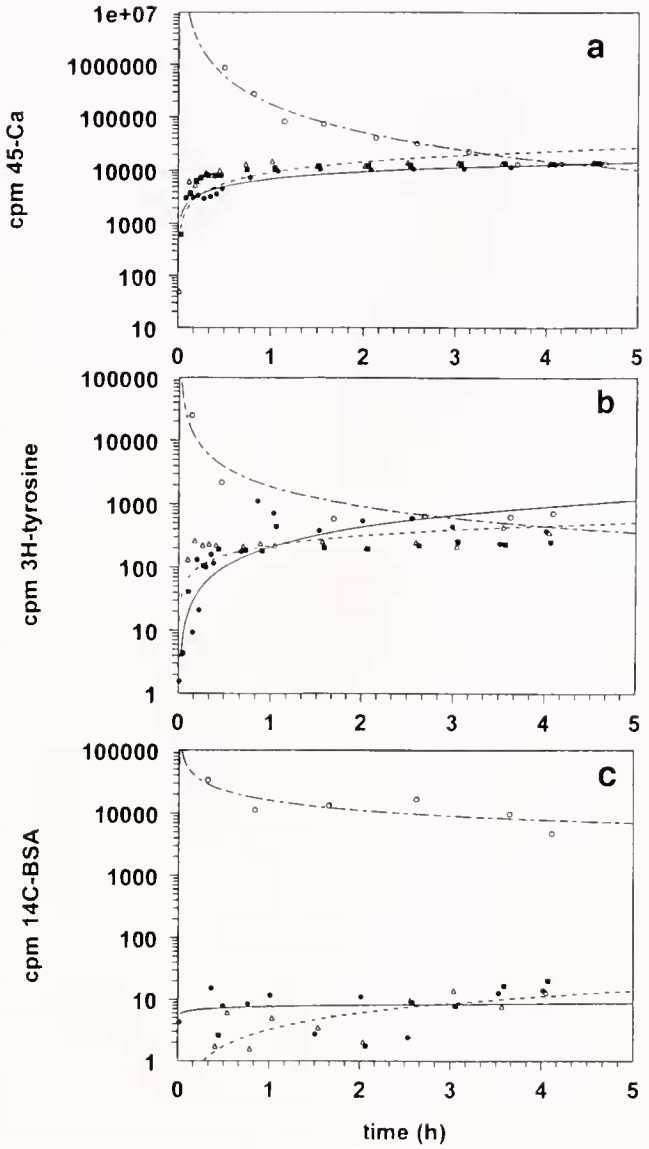

Figure 1. Radioactivity (cpm) in *Mercenaria mercenaria* extrapallial fluid (EPF) and blood following injection of (a) ^{45}Ca (MW = 40), (b) ^{3}H-tyrosine (MW = 181), and (c) ^{14}C-BSA (MW = 66 kDa) into the right shell EPF. Extrapallial fluid samples were drawn from the central zone of the right (— ○ —) and left (— ● —) shells; blood samples were drawn from the anterior (. . .△. . .) and posterior (--■--) adductor muscles. Lines were drawn by a logarithmic-fit function using SlideWrite Plus® V4.0.

Furthermore, ^{14}C-BSA concentration in the left shell EPF appeared to remain constant over the 4-h period. The time required for the introduced BSA to become homogeneous with the blood was estimated to be at least 22 h.

A confirmatory experiment using nonlabeled BSA was performed to determine whether the BSA remained solubilized in EPF or became adsorbed to the mantle tissue during the course of the experiment. SDS-polyacrylamide gels of EPF and blood plasma showed that the 66-kDa band of BSA was present only in the right shell EPF (the point of introduction) in samples taken 3 h and 6 h post-injection (results not shown). Staining intensities of the bands at 3 and 6 h were similar, indicating that the BSA was not bound to tissues. If BSA was present in the left shell EPF and in the plasma samples at 3 and 6 h, it was below the detection limits of SDS-PAGE.

The shapes of the semilog plots in Figure 1 indicate a rapid decrease in injected counts during the first 30 min, followed by a longer phase of gradual decline and attainment of an apparent steady state over the next 4–4.5 h. This suggests that the release of radiolabel from the EPF may be biphasic. Apparent depuration constants (k_ds) were calculated from the slopes of tangents to the initial and final portions of the plots. Depuration constants for the rapid phase (k_{d1}) were estimated as 9.7, 12.0, and 2.4 h^{-1} for ^{45}Ca, ^{3}H-tyrosine, and ^{14}C-BSA, respectively, whereas constants for the slow phase (k_{d2}) were 0.1, 0.08, and 0.04 h^{-1}. Biological half-lives calculated from the k_{d2} values were 6.9 h for ^{45}Ca, 8.7 h for ^{3}H-tyrosine, and 17.3 h for ^{14}C-BSA.

The mean concentration of total Ca measured in the blood plasma of 10 quahogs was 10.9 ± 1.3 m*M*, of which 0.4 ± 0.1 m*M* was free Ca^{2+}. Bound Ca was estimated as 10.5 ± 1.2 m*M*. Equilibrium dialysis of the plasma samples revealed that the majority of bound Ca (7.2 m*M*; Table I) was nondialyzable (*i.e.*, bound to ligands >1000 Da), but 1.1 m*M* was dialyzable, and 0.2 m*M* was free Ca. These concentrations reflect a 78% recovery of total Ca following dialysis; the remainder was presumably bound to the walls of the dialysis bags. Expressed as percentage of total Ca recovered, bound Ca (nondialyzable and dialyzable) and free Ca^{2+} amounted to 84.7, 12.9, and 2.4%, respectively (Table I).

Inorganic Ca speciation obtained by MINTEQA2 predicted that a high proportion of plasma Ca should be present as free Ca^{2+} (78.9%; Table II, permutation 1), and the remainder as bound Ca (primarily $CaSO_4$, 20.2%). However, this prediction is inconsistent with the values of 2.4% free Ca^{2+} and 12.9% bound-dialyzable Ca obtained by the dialysis experiments (Table I). If the SO_4^{2-} concentration used in model permutation 1 (46.1 m*M*, Crenshaw, 1972a) was replaced by the seawater SO_4^{2-} concentration (28.2 m*M*, Mantoura *et al.*, 1978), the distribution of $CaSO_4$ was reduced to 13.1% and 12.5% for plasma and EPF, respectively (Table II, permutation 2). The theoretical prediction of free Ca^{2+} (85.9% in plasma) was even further out of line with the measured Ca^{2+} concentrations.

Inclusion of Ca-binding organic ligands (*e.g.*, proteins) in the milieu is a way to reconcile this discrepancy with free Ca^{2+}. Serum albumin in vertebrate blood, for instance, binds free Ca with an affinity constant ($\log_{10} K_a$) of 2 M^{-1} and a binding capacity of 16 Ca ions per molecule

Table I

Distribution of calcium in Mercenaria mercenaria *blood plasma*

	Concentrations in m*M*		Percentage of total Ca recovered	
Ca	Nondialyzyable	Dialyzable	Nondialyzable	Dialyzable
Bound*	7.2 ± 0.9	1.1 ± 0.2	84.7 ± 10.6	12.9 ± 2.4
Free (Ca^{2+})†	nd	0.2	nd	2.4

Note: Values expressed as mean ± SD, $n = 10$. Results were obtained by equilibrium dialysis.
* Represents the difference between total Ca and free Ca^{2+}.
† nd = not detected.

(Putnam, 1975). A hypothetical Ca-binding protein with 15 independent Ca-binding sites per molecule was included in the model, and the resulting Ca speciation was tested under a range of concentrations (0.1 to 100 m*M* protein) and affinity constants ($\log_{10} K_a = 2-6\ M^{-1}$). Free Ca distributions of less than 2.5% were obtained in the 10–100 m*M* range at affinity constants 2 and 3 M^{-1}, and in the 0.1–10 m*M* range at K_as 4–6 M^{-1} (Fig. 2). For instance, 60 m*M* protein with a log K_a of 2.1 M^{-1} resulted in 2.3% free Ca^2 and 96.7% Ca-protein complex in the plasma (Table II, permutation 3). If a 1:5 protein:Ca ratio were modeled, three times as much protein would be required to bind the same amount of Ca.

SDS-PAGE was carried out to compare the protein profiles of plasma and EPF. Seventeen distinct bands ranging in size from 30 to greater than 230 kDa could be detected in EPF by Coomassie brilliant blue staining (Fig. 3). Blood plasma, on the other hand, revealed 15 bands, 5 of which (MW = 157, 125, 42, 35, and 34 kDa) stained faintly but were detected by scanning densitometry (results not shown). Two prominent protein bands (MW = 74 and 51 kDa) were observed only in the EPF. These band patterns were consistent in samples from four quahogs, although the intensity of staining varied between animals. Four bands in the EPF (MW = 212, 157, 142, and 74 kDa) and two in plasma (MW = 212 and 142 kDa) stained positive for glycoproteins by the highly sensitive periodic acid-silver staining technique.

Discussion

Results of the tyrosine circulation experiment indicate that small organic compounds, in the range of a few hundred daltons, are probably freely exchanged between the EPF and the blood. These compounds behave like Ca with respect to their rate of transport across the outer mantle epithelium. A size increase of about 2 orders of magnitude may, however, drastically limit the passage of a molecule across this epithelium. Bovine serum albumin (MW = 66 kDa), for example, was not rapidly redistributed with the blood. From the cold BSA injection experiment, it was apparent that BSA was retained in the EPF in solution, and not taken up by or bound to the outside of the underlying tissue. Similarly, the SDS-PAGE results identified two protein bands in the EPF (MW > 50 kDa) that were not detected in the plasma, indicating that the outer mantle epithelium contains an effective barrier to at least some high molecular weight proteins. These observations are consistent with those of Bielefeld *et al.* (1993), who found the outer mantle epithelium of the

Table II

Theoretical speciation of calcium in Mercenaria mercenaria *blood plasma and extrapallial fluid*

Model permutation*	Sample	Ca^{2+}	$CaSO_4$	$CaHCO_3^+$	$CaOH^+$	Ca-Protein
1	Plasma	78.9	20.2	<0.9	trace	0
	EPF	85.9	19.3	<0.9	trace	0
2	Plasma	85.9	13.1	<0.9	trace	0
	EPF	86.5	12.5	<0.9	trace	0
3	Plasma	2.3	1.0	0	0	96.7
	EPF	2.2	0.9	0	0	96.9

Note: Values expressed as percentage of total calcium. Results were obtained using the equilibrium speciation model MINTEQA2 (Allison *et al.*, 1991).
* Premutations: (1) concentrations of inorganic ions taken from Crenshaw (1972a); (2) sulfate concentration decreased from 46.1 to 28.2 m*M*; (3) 60 m*M* of hypothetical Ca-binding protein (15:1 Ca:protein) with $\log_{10} K_a$ of 2.1 M^{-1} added.

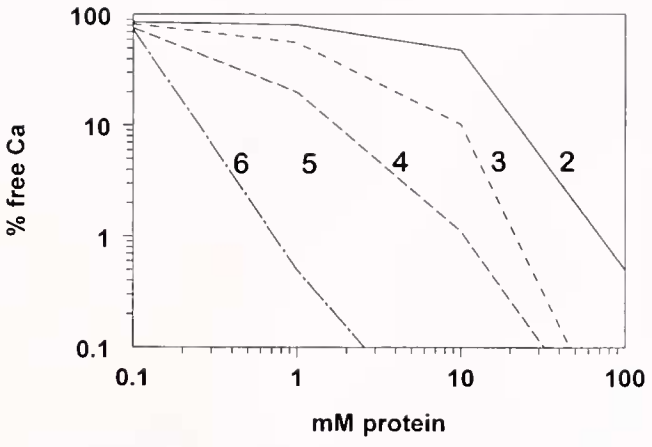

Figure 2. Percentage distribution of free Ca (ionic Ca^{2+}) in the blood plasma of *Mercenaria mercenaria* in the presence of a hypothetical Ca-binding protein with 15 Ca-binding sites. The lines represent a range of binding affinity constants tested ($\log_{10} K_a$ = 2-6 M^{-1}). Data were generated using the equilibrium speciation model MINTEQA2 (Allison *et al.*, 1991).

snail *Biomphalaria glabrata* impermeable to injected horseradish peroxidase (MW = 40 kDa).

An important factor governing the direction of Ca flux across the outer mantle epithelium is anaerobiosis. Anaerobiosis induced upon shell closure is accompanied by redissolution of $CaCO_3$ from the shell to maintain a more uniform plasma pH through the bicarbonate buffering system (Crenshaw and Neff, 1969; Hudson, 1992; Littlewood and Young, 1994). This leads to a net Ca flux away from the central zone EPF. On the other hand, injury to the shell causes remobilization of Ca into the EPF. Calcium for shell repair is derived predominantly from the calcium cells of the mantle and foot (see Watabe, 1983), by dissolution of $CaCO_3$ and $Ca_3(PO_4)_2$ spherules. Because the quahogs in our experiments had damaged shells and were kept out of water for as long as 4.5 h, the 3-h timeframe for Ca exchange in this study probably reflected opposing Ca fluxes resulting from anaerobiosis and attempts at shell repair.

Other studies have found similar timeframes for Ca exchange in bivalves. ^{45}Ca introduced in the seawater took 2 h to reach a steady state with the mantle in *Argopecten irradians* (Wheeler *et al.*, 1975), and Crenshaw and Neff (1969) detected radioactivity in the EPF of *M. mercenaria* 2.75 h after ^{45}Ca was added to the seawater. *Scrobicularia plana* showed rapid shell dissolution about 2 h after the onset of salinity stress (Akberali, 1980). These results indicate that the timeframes involved in both inward Ca flux (for shell deposition and repair) and outward flux (for shell redissolution due to salinity and anaerobic stress) may be similar. The total Ca concentration for blood plasma found in this study (10.9 m*M*) is similar to reported values for *M. mercenaria* (10.5 m*M*; Crenshaw, 1972a) and *Mytilus edulis* (12.6 m*M*; Bayne, 1976). Knowledge of total Ca concentrations, however, is of little value in understanding the processes involved in shell formation. These processes can only be inferred in light of Ca speciation (*i.e.*, the distribution of ionic Ca^{2+}, inorganic Ca species, and organically bound Ca chelates). Shell deposition in molluscs occurs only when Ca^{2+} and CO_3^{2-} are present in concentrations exceeding their solubility products (Wilbur and Saeluddin, 1983). Whereas CO_3^{2-} is derived from both the external medium and metabolic CO_2 (Dillaman and Ford, 1982), Ca must be obtained externally, and transported via the blood to the EPF. Different Ca species may be involved in each step of this process.

The MINTEQA2 model predicts the distribution of free and bound Ca in blood plasma as 78.9% and 21.1%, respectively. However, this prediction is inconsistent with the 2.4% free Ca and the 12.9% bound-dialyzable Ca obtained by the dialysis experiments. The model-derived figure of 20.2% $CaSO_4$ is probably an overestimate of inorganic Ca, because the SO_4^{2-} concentration (46.1 m*M*)

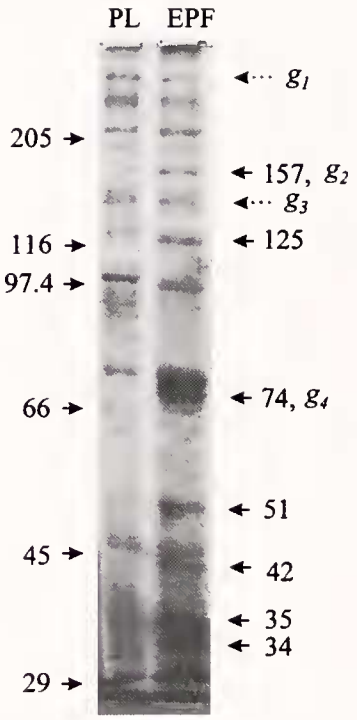

Figure 3. Comparison of *Mercenaria mercenaria* blood plasma (left lane) and extrapallial fluid (EPF; right lane) proteins (~50 μg/lane) by 7.5% SDS-PAGE stained with Coomassie brilliant blue. The positions of molecular weight markers (kDa) are indicated on the left; 7 protein bands predominant in the EPF are marked on the right. Bands that stained positive as glycoproteins by periodic acid-silver staining are indicated as g_1–g_4. Bands g_1 (212 kDa) and g_3 (142 kDa) were detected in both plasma and EPF.

reported by Crenshaw (1972a) was obtained by hydrolysis of ester sulfates, and therefore is at least partly organic in nature. Replacing Crenshaw's SO_4^{2-} concentration with that of seawater SO_4^{2-} reduces $CaSO_4$ to 13.1%, a figure almost identical (coincidentally) to the bound-dialyzable fraction. However, the high percentage of Ca^{2+} predicted by MINTEQA2 is still not explained.

It is apparent that inorganic speciation alone does not explain the very low percentage of free Ca^{2+} obtained experimentally. Calcium-binding organic ligands are the only species that can plausibly account for this difference. If analogous to vertebrate blood (Putnam, 1975; Scott and Bradwell, 1983), Ca-binding proteins in quahog plasma and EPF probably exhibit nonspecific, low-affinity binding with high binding capacities. Although the molar concentrations and affinity constants (K_a) of quahog Ca-binding proteins are not known, inclusion of a single protein (1:15 Ca binding, with a log K_a of 2.1 M^{-1}) in the MINTEQA2 model resulted in free Ca distributions similar to those obtained by dialysis. Although the assumption of a single protein is simplistic, these binding characteristics compare well with Ca binding by serum albumin. Organic matrices of bivalve shells possess both somewhat high-affinity (log K_a = 4–5 M^{-1}) and low-affinity (log K_a = 3–4 M^{-1}) Ca-binding components (Wheeler and Sikes, 1984).

Interestingly, the contribution of SO_4^{2-} to Ca speciation became negligible whenever proteins were added and free Ca was reduced below 5%. This suggests that $CaSO_4$ is not a major Ca species in plasma and EPF, and that the 12.9% bound-dialyzable fraction obtained by dialysis consists primarily of low molecular weight organic Ca-binding ligands. Such ligands, including amino acid anions, carboxylate, salicylate, and ascorbate, are found in blood plasma, and are known to bind a variety of cations (May *et al.*, 1977). Taken together, these results indicate that *M. mercenaria* has a multi-component Ca transport system consisting of low molecular weight ligands as well as one or more proteins. This is consistent with a previous finding by Misogianes and Chasteen (1979) for *Mytilus edulis* EPF.

Because of the predominance of organically bound Ca in quahog plasma, one must wonder about the sequence of events following our injection of $CaCl_2$ into the EPF of the quahog. In all likelihood, the injected Ca was rapidly reproportioned among a variety of inorganic and organic ligands, with most of the Ca binding to EPF proteins. Because similar proteins are also present in the plasma, any free Ca initially entering the plasma would also be bound to proteins, and subsequently equilibrated with tissue Ca. Calcium binding in EPF could actually be slightly greater or stronger than Ca binding to plasma proteins, because five proteins present in the EPF are not prominent in the plasma, and two are absent. The results of our gel electrophoresis indicate that any EPF Ca-protein complexes with a molecular weight greater than about 30 kDa would translocate to the plasma slowly. Yet we observed a rapid flux of ^{45}Ca between the EPF and the plasma (with an apparent steady state established within 3 h). Therefore, the binding of Ca with these EPF proteins must be relatively weak, in agreement with MINTEQA2 modeling. Weak binding would allow a rapid exchange of Ca among all of the possible inorganic and organic species present in blood and EPF, and would explain how Ca can be translocated rapidly while the larger molecular weight proteins are exchanged much more slowly.

The prominent 157-kDa glycoprotein band seen in the EPF is similar in size to a 160-kDa protein isolated from the soluble shell matrix of *M. mercenaria* by Crenshaw (1972b). This matrix protein also stained positively with the glycoprotein-specific stains ponceau S and alcian blue, and is believed to bind Ca and help in $CaCO_3$ crystal nucleation. Similar calcium-binding proteins have been isolated from the EPF and shell matrices of other bivalves (Samata, 1990; Donachy *et al.*, 1992). Shell injury triggers the outer mantle epithelium to secrete glycoproteins, mucoproteins, and mucopolysaccharides into the EPF (Timmermans, 1973; Meenakshi *et al.*, 1975; Watabe, 1983). The 74- and 51-kDa protein bands we found exclusively in the EPF could represent components of these secretions.

In summary, the experiments conducted in this study demonstrate *in vivo* that Ca and small organic molecules are readily exchanged between the EPF and blood of *M. mercenaria*. The passage of macromolecules across the outer mantle epithelium is restricted, and extrapallial fluid and plasma differ in their protein compositions. Inorganic speciation modeling predicts that concentrations of free Ca^{2+} in plasma and EPF are vastly higher than those obtained experimentally. This discrepancy can be overcome by including organic ligands in Ca speciation models for both biological fluids. Most of the Ca in blood plasma and EPF is bound to macromolecules, and the remainder to small organic ligands. Calcium is transported by a system of one or more relatively weakly binding proteins and smaller organic molecules.

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